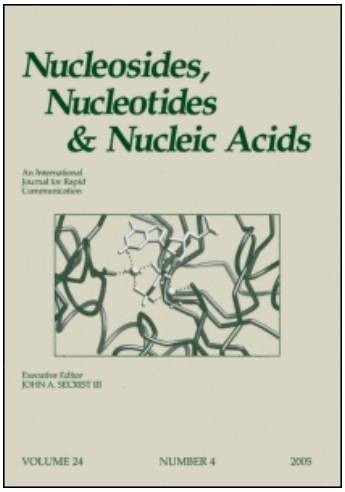

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S. Carriero[a]; R. S. Braich[a]; R. H. E. Hudson[b]; D. Anglin[c]; J. D. Friesen[c]; M. J. Damha[a]
[a] Department of Chemistry, McGill University, Montreal, Canada [b] Dept. of Chemistry, University of Western Ontario, London, Canada [c] Banting and Best Institute, Dept. of Medical Research, University of Toronto, Toronto, Canada

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INHIBITION OF *IN VITRO* PRE-mRNA SPLICING IN *S. CEREVISIAE* BY BRANCHED OLIGONUCLEOTIDES

S. Carriero,[1] R. S. Braich,[1] R. H. E. Hudson,[*] D. Anglin,[2] J. D. Friesen,[2] and M. J. Damha[1,†]

[1]Department of Chemistry, McGill University, Montreal, QC, Canada, H3A 2K6
[2]Banting and Best Institute, Dept. of Medical Research, University of Toronto, Toronto, ON, Canada, M5G 1L6

ABSTRACT

A series of V- and Y-shaped nucleic acids, related to the splicing intermediates derived from *S. cerevisiae* actin pre-mRNA, were prepared. The effects of such branched nucleic acids (bNAs) on the efficiency of *in vitro* pre-mRNA splicing in yeast were studied. The exogenous bNAs each effect the efficiency of splicing, yet to different degrees, depending on the sugar composition and topology of the molecules. Y-shaped RNAs inhibited the formation of mRNA (*i.e.* RNA splicing) to the greatest extent.

The post-transcriptional processing or "splicing" of precursor messenger RNA (pre-mRNA) is an intricate process involving a number of stable *trans*-acting factors (RNA-RNA, RNA-protein and auxiliary proteins) which constitute the spliceosome (1). Branched nucleic acids (bNAs) containing vicinal 2′, 5′ and 3′, 5′ phosphodiester linkages have been of particular interest since they were first discovered as lariat intron splicing intermediates in HeLa cells (2). During the splicing process, pre-mRNA undergoes two concomitant phosphodiester transfer reactions

[*]Current address: Dept. of Chemistry, University of Western Ontario, London, ON, Canada, N6A#5B7.
[†]Corresponding author.

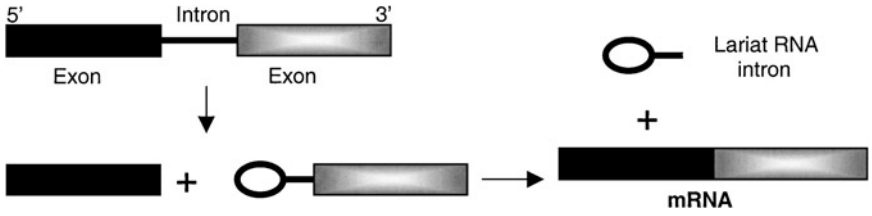

Figure I. Splicing of precursor messenger RNA (pre-mRNA) proceeds via two transesterification reactions. In the first step, the pre-mRNA is cleaved producing a lariatexon-3′ intermediate (gray) and the 5′-exon (black). In the second step, the exons are ligated and lariat intron excised, leading to the formation of mRNA. The branchpoint of the lariat structure consists of an adenosine nucleotide containing vicinal 2′,5′ and 3′,5′-phosphodiester linkages. The synthetic Y-shaped RNA molecules prepared in this study mimic the branchpoint of the lariat RNA structure.

(3) resulting in the generation of the mature mRNA transcript, via exon ligation, and the release of the RNA lariat intron (Fig. I). *In vivo*, the lariat RNA is rapidly debranched and degraded by host cellular nucleases but is reasonably stable and may be observed in reactions *in vitro* (4).

The splicing apparatus in both yeast and mammalian cells appear to be highly conserved, however, the sequences surrounding the splice sites in yeast pre-mRNA show only some sequence similarity with those in man (5). In budding yeast, the excised intron lariats contain the invariant branch point sequence 5′-UACUA$\underline{A}^{2'GU-}{}_{3'C-}$, ($\underline{A}$ is the branch point nucleotide) whereas in higher order eukaryotes, stringent sequence conservation surrounding the branch point adenosine nucleotide is not observed [5′-YNYUR$\underline{A}^{2'GU-}{}_{3'Y-;}$ R=Pu and Y=Py]. The specific events required for the recognition of this conserved branch point structure during splicing are not completely understood (6,7). Synthetic bNAs, related to the splicing intermediates derived from *S. cerevisiae* actin pre-mRNA, serve as useful probes to investigate branch point recognition in yeast nuclear extract capable of performing *in vitro* pre-mRNA splicing. Using our published protocols on bRNA synthesis, a variety of potential substrates with symmetrical 2′ and 3′ sequences were synthesized in order to assess bNA recognition during *in vitro* pre-mRNA splicing (Table I) (8). Since the 2′ and 3′ sequences of the synthetic bNAs are identical, the branched core architecture is different from that of the yeast consensus sequence (*i.e.* 5′..A$\underline{A}^{2'G..}{}_{3'G..}$ *vs.* 5′..A$\underline{A}^{2'G..}{}_{3'G..}$). However, this may be inconsequential as studies on the branch point sequences in a yeast intron demonstrate that mutation of the conserved 3′-C nucleotide to G has no discernable effect on splicing (9).

Yeast splicing extracts and the ^{32}P internally radiolabelled actin pre-mRNA transcript were prepared according to the method of Lin and co-workers (10). Extracts were prepared from the wild type cell W3031A (*MAT*a, *ade2*-1, *his3*-11, *15 leu2*-3, *112 trp1*-1, *ura3*-1, *can1*-100). The transcript was synthesized using an *Eco*R1-linearized pGEM.gzf(-) DNA as template as described. *In vitro* pre-mRNA splicing reactions were conducted according to the method of Ghetti and co-workers and in the presence of 1–100 μM of linear, V-shaped and Y-shaped oligonu-

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Table I. Linear and Branched Oligonucleotide Sequences Used in *In Vitro* Pre-mRNA Splicing Assays

	SEQUENCE[a]	TOPOLOGY
1	5′-A(2′-5′GTA TGT, 3′-5′GTA TGT)	V-DNA
2	5′-TAC TAA(2′-5′GTA TGT, 3′-5′GTA TGT)	Y-DNA
3	5′-AUGGAUUCUGAUAUGUUCUA-3′	Linear A-RNA
4	5′-AUGGAUUCUGGUAUGUUCUA-3′	Linear G-RNA
5	5′-A(2′-5′GUA UGU, 3′-5′GUA UGU)	V-RNA
6	5′-UAC UAA(2′-5′GUA UGU, 3′-5′GUA UGU)	Y-RNA
7[b]	5′-*c*UAC UAA(2′-5′GUA UG*ccc*, 3′-5′GUA UG*ccc*)	Y-RNA

[a]Sequences purified by preparative polyacrylamide gel electrophoresis (24%/ 7M urea), and desalted by size exclusion chromatography on Sephadex G-25®.
[b]*c* = L-2′-deoxycytidine (L-dC).

cleotides (11). By using a short reaction time (*ca.* 30 min.), the actin pre-mRNA, lariat-containing intermediates, and mature mRNA are readily separated and identified by denaturing polyacrylamide gel electrophoresis and quantitated by phosphorimaging (Fig. IIa). The effects of the bNAs on splicing efficiency were measured by quantitating the amount of mature mRNA produced as a percentage of the total radioactivity in each lane, and normalized with respect to the reaction lacking any bNA (0 μM). As shown in Figure IIb, the exogenous DNA and RNA each effect the efficiency of splicing, yet to different degrees. The synthetic Y-shaped RNA **6** inhibited the formation of the mature RNA product to a much greater extent than DNA **2**, *i.e.* reduction of 97% at 10 μM *vs.* 75% at 95 μM, respectively.

Radioisotope (^{32}P) 5′-end labeling studies of the branched synthetic oligonucleotides of interest have shown partial degradation of the substrates, presumably by exonucleases present in the yeast nuclear extract. The incorporation of unnatural L-2′-deoxycytidine (L-dC) nucleotides at the ends of an otherwise unmodified D-oligomer provides adequate stability towards exonuclease hydrolysis (12). Sequence **7** was synthesized for this reason, as well as to determine the lowest concentration limit required for maximal inhibition of splicing by Y-RNA substrates. Remarkably, at a concentration of 3 μM, **7** reduced splicing efficiency by as much as *ca.* 85%.

Similar results were found for V-RNA, **5**, as compared with V-DNA, **1**. Both substrates inhibit mRNA formation to the same extent at high concentra-

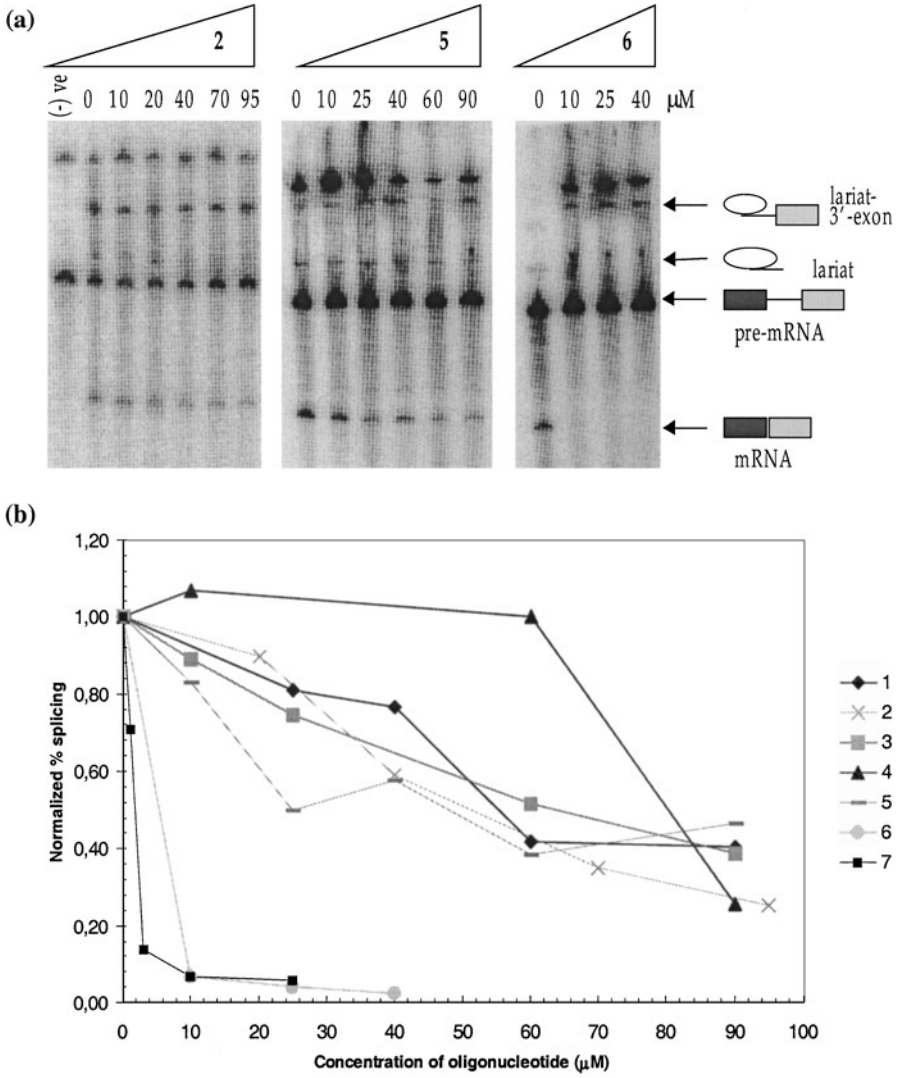

Figure II. (a) Autoradiogram of PAGE analysis of *in vitro* pre-mRNA splicing reactions in the presence of synthetic oligonucleotides. The negative control ((-)ve) is actin pre-mRNA. (b) Measured splicing efficiency reduction for bNA substrates 1–7 (see Table 1).

tion (90 μM), but at lower concentrations (25 μM), mRNA formation is reduced to *ca*. 20% with **1**, and 50% with **5**. In addition, preliminary investigations on splicing inhibition by branched oligothymidylates (13) demonstrate the requirement for a well-defined branch point (5′,2′, and 3′ tails) in order to achieve maximal reduction in splicing efficiency. The results suggest that branched RNA oligonucleotides preferentially inhibit splicing through the recognition and sequestering of an unknown spliceosomal element, possibly a RNA-binding protein (14). Experiments with linear oligoribonucleotides (**3** and **4**, Fig. IIb) were conducted to determine the role

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of sequence-specific effects in splicing inhibition. At low concentrations, splicing inhibition is minimal, but at the highest concentration studied (90 μM), **3** and **4** reduce splicing efficiency by *ca*. 70% and 60%, respectively. Similar results were previously obtained with linear oligothymidylates and may represent some non-specific effects, although more studies are needed to confirm this. It is speculated that the putative branched recognition factor(s) may have a low affinity for linear sequences, and higher concentrations are required in order to sequester them from the splicing machinery.

The results reported herein demonstrate the utility of synthetic bNAs in the study of branch point recognition *in vitro*. Furthermore, synthetic bNAs may prove to be invaluable tools for the separation and purification of the putative branch point recognition factor through affinity selection studies.

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